ENGLISH TRANSLATION OF KOREAN PATENT APPLICATION NO. 10-2001-0033399

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I AM A TRANSLATOR OF HMP P.C., AND DECLARE THAT I PREPARE THIS TRANSLATION AND IT IS TRUE ENGLISH TRANSLATION MADE FROM KOREAN PATENT APPLICATION NO. 10-2001-0033399

At Seoul, Korea on August 2, 2006

Chang Jae Woo

[ABSTRACT]

The present invention relates to a protein having immune-enhancing, anti-tumor and anti-angiogenic effects. More particularly, it relates to a p43 having cytokine activity, anti-tumor activity and anti-angiogenic activity and comprising the amino acid sequence set forth in SEQ ID NO:2.

The p43 of the present invention effectively induces cytokines to increase immune responses, and effectively inhibit tumor and angiogenesis, so that it can be effectively used in the immune-enhancing agent, anti-tumor agent or anti-angiogenic agent as an active ingredient.

[REPRESENTATIVE DRAWING]

Figure 1

[KEY WORDS]

p43, anti-cancer, angiogenesis, immune-enhancing

[SPECIFICATION]

[TITLE OF THE INVENTION]

p43 Protein Having Immune-Enhancing, Anti-Tumor And Anti-Angiogenic Activities

[BRIEF DESCRIPTION OF THE DRAWINGS]

Fig. 1 illustrates results of SDS-PAGE analysis of the full-length p43, and N-terminal protein and C-terminal protein of p43.

Fig. 2 illustrates the expression induction of TNF-α, IL-6 and IL-8 by p43 and EMAP II.

Fig. 3 illustrates the secretion of MMP-9(matrix metalloprotease-9) by p43 and EMAP II.

Fig. 4 illustrates a graph showing results of in vitro motility assay of p43 and EMAP II for human melanoma cells.

Fig. 5a illustrates pictures by a microscope showing results of the angiogenesis induction after treatment of bovine aorta endothelial cells with p43 at 100 nM.

Fig. 5b illustrates a graph showing results of the angiogenesis induction after treatment of bovine aorta endothelial cells with each of the full-length p43(F), p43 N-terminal peptide(N) and p43 C-terminal peptide(C) at 100 nM.

Fig. 5c illustrates results of DNA fragmentation assay for the apoptosis induction after treatment of bovine aorta endothelial cells with p43 at 100 nM.

Fig. 5d illustrates results of caspase-3 activity for the apoptosis induction after treatment of bovine aorta endothelial cells with p43 at 100 nM.

[TECHNICAL FIELD AND BACKGROUND ART]

The present invention relates to a protein having immune-enhancing, anti-tumor and anti-angiogenic effects. More particularly, it relates to a p43 having cytokine activity, anti-tumor activity and anti-angiogenic activity and comprising the amino acid sequence set forth in SEQ ID NO:2.

BACKGROUND

Endothelial monocyte activating polypeptide II (EMAP II) is a polypeptide isolated from the methylcholathrene A-transformed fibrosarcoma cells (Kao et al., *J. Biol. Chem.* 267:20239-20247, 1992), and is known to suppress primary and metastatic tumor growth and trigger apoptosis in proliferating endothelial cells (Schwarz et al., *J. Exp. Med.* 190:341-353, 1999).

The EMAP II is released from its precursor, p43, upon apoptosis. Its precursor, p43 (pro-EMAP II), consists of 312 amino acids, and is normally associated with the multi-tRNA synthetase complex (Park et al., *J. Biol. Chem.* 274:16673-166776, 1999). However, it is cleaved by the activated caspase-7 in apoptotic condition to separate its C-terminal domain and produce EMAP II (Behrensdorf et al., *FEBS Lett.* 466:143-147, 2000).

The sequence of EMAP II shows homology to the domains present in several different aminoacyl-tRNA synthetases (Quevilon et al., *J. Biol. Chem.* 272:32573-32579, 1997) and could bind tRNAs. Additionally, the N-terminal synthetic 15 amino acids peptide was shown to be responsible for the cytokine activities of EMAP II (Kao et al., *J. Biol. Chem.* 269:9774-9782, 1994).

The structure and maturation of EMAP II are similar to 14.5 kDa IL-lb that is associated with a immune response. The Il-1b cytokine is generated by the cleavage of

ICE (caspase-1) from its 33 kDa inactive precursor (pre-IL-1b). Although the maturation processes of the two cytokines are similar, the precursor of EMAP II, p43, appears to be different from pré-IL-1b in that it is associated with the multi-tRNA synthetase.

As such, the EMAP II domain has been extensively studied for its cytokine activities, and the prevent inventors have previously shown that the N-terminal domain of p43 is responsible for the interaction with the N-terminal non-catalytic extension of human arginyl-tRNA synthetase, and both of the domains in p43 are required to give a stimulatory effect on the interacting arginyl-tRNA synthetase (Park et al., J. Biol. Chem. 274:16673-16676, 1999).

Additionally, US Patent No. 5,641,867 disclosed anti-tumor activity of EMAP II.

The present inventors compared p43 with EMAP II for various cytokine activities, anti-tumor activity and anti-angiogenic activity in the present invention and found that p43 itself exerts more potent cytokine activity, anti-tumor activity and anti-angiogenic activity than its proteolytic product, EMAP II.

[THE OBJECTS OF THE INVENTION]

Accordingly, it is an object of the invention to provide an immune-enhancing agent containing p43 as an active ingredient.

Another object of the invention is to produce anti-tumor and anti-angiogenic agents containing p43 as an active ingredient.

[DISCLOSURE OF THE INVENTION]

To accomplish the object of the invention, the present invention provides an immune-enhancing agent containing p43 having the amino acid sequence of SEQ ID NO:2 as an active ingredient.

To accomplish another object of the invention, the present invention provides the anti-tumor and anti-angiogenic agents containing p43 having the amino acid sequence of SEQ ID NO:2 as an active ingredient.

Hereinafter, the present invention will be described in detail.

The present invention relates to the immune-enhancing, anti-tumor and anti-angiogenic agents containing p43 having the amino acid sequence of SEQ ID NO:2 as an active ingredient.

In the present invention, in order to verify that p43 itself has improved immune-enhancing activity, and also shows anti-tumor and anti-angiogenic activities, cytokine activity, anti-tumor activity and anti-angiogenic activity of p43, its C-terminal and N-terminal peptides were investigated.

Thus, in the present invention, these three proteins were expressed, separated and purified according to the conventional method (Park et al., *J. Biol. Chem.* 274:166673-166676, 1999). A vector for gene transformation is, but are not limited to, preferably pET28a(Novagen). Because a plurality of sequences encoding histidine are present in the vector, histidine is expressed together when protein is expressed, thus easily purifying the protein.

In one preferred embodiment of the present invention, immune-enhancing activity of p43 and EMAP II as its C-terminal region were determined. The immune-enhancing activity is determined by analyzing the amount of various kinds of cytokines which are induced by p43 and EMAP II. Particularly, in the present invention, the immune-enhancing activity is determined by analyzing the amounts of TNF-α, IL-8 and IL-6. These three cytokines are produced from mainly macrophages, and are known to be associated with immune responses by changing properties of other cells. In the

present invention, it was determined that p43 showed higher promotion of induction of three cytokines than EMAP II when p43 and EMAP II are treated with the same concentration of the cytokines, and p43 itself had more effective immune-enhancing activity.

In another preferred embodiment of the present invention, MMP-9(matrix metalloprotease-9) inducing activity of p43 was determined. The MMP-9 inducing activity was determined by the conventional method in the art. In particular, in the present invention, the MMP-9 inducing activity is determined by performing gelatin zymography. The MMP-9 is a protein decomposing the extracellular matrix, and is known to participate metastasis and induce TNF-α. Additionally, secretion of MMP-9 promotes secretion of angiostatin that is known as a material inhibiting angiogenesis. As shown in Fig. 3, the amount of MMP-9 was more increased by p43 than EMAP II. Thus, p43 itself has more effective immune-enhancing activity and anti-angiogenic activity.

Additionally, in another preferred embodiment of the present invention, the chemotaxis effect of p43 and EMAP II was determined by treating a melanoma cell with p43 and EMAP II separately. The chemotaxis effect may be determined by the conventional method. In the present invention, the chemotaxis effect was determined by the in vitro motility test. Generally, if the chemotaxis activity is high, cancer cell specific lymphocytes, nonspecific neutrophils or macrophages is induced to the cancer cells, thus promoting anti-tumor activity. As shown in Fig. 4, it was observed that the chemotaxis activity was high when p43 was treated. Thus, it is shown that p43 is an effective chemoattractant, and acts as an anti-tumor agent.

Meanwhile, in order to verify whether p43 inhibits angiogenesis, apoptotic activity of p43 was determined in an endothelial cell, particularly, bovine aorta endothelial cell in one preferred embodiment of the present invention. The apoptotic activity of p43 may be determined by the conventional method in the art. In the present

invention, the apoptotic activity of p43 was determined by morphology analysis of cells, DNA fragmentation assay and caspase-3 activity measurement. As shown in Figs. 5a to 5d, it was observed that p43 inhibited the growth of endothelial cells and induced apoptosis, and the caspase activity was high. The caspase-3 is a protein inducing apoptosis in cells. Thus, the high caspase-3 activity indicates progression of apoptosis within a short period.

The immune-enhancing agent, anti-tumor agent or anti-angiogenic agent comprising p43 as an active ingredient of the present invention may be administered into human or animals in any one type applicable to human or animals, preferably a type supported by a carrier.

The carrier is at least one selected from solid, semi-conductor, liquid diluent, filler and other preparation adjuvants and is used in the amount of 0.1-99.55%. The therapeutic agent may be administered orally or non-orally. The non-oral administration may be topical, subcutaneous, intramuscular, arterial or intravenous administration, and rectal administration. The administration type suitable for the administration pathways may be prepared with the conventional tools.

The oral administration is performed by using a solid or liquid unit administration type, for example, bulk powder, powder, granule, tablet, capsule, syrup and suspension. If necessary, the unit administration type of the oral administration may be micro-capsulated. The further coating of the therapeutic agent or inclusion of active ingredients into the polymer or wax allows to extend the active period and to obtain sustained-release ability.

The dosage of the therapeutic agent may be preferably determined by considering age, body weight, administration pathway, disease and its severity, and other factors of a patient.

Additionally, the present inventors identified the three dimensional structure of EMAP II as the C-terminal region of p43 through X-ray crystallography, and confirmed that the EMAP II structure consisted of 11 β-strands and 3 α-strands. The final model contains two molecules per asymmetric unit (residues 3-166) and 193 water molecules, refined at 1.8 Å resolution.

The EMAP II structure consists of 11 ß -strands forming a structural core and 3 flanking α-helices: the strands ß1 (residues 10-21), ß2 (residues 28-34), ß3 (residues 40-46), ß4 (residues 59-66), ß 5i (residues 70-72), ß 6i (residues 75-77), ß7 (residues 79-85), ß8 (residues 90-92), ß 9 (residues 103-106), ß 10 (residues 132-134), ß 11 (residues 140-142), and the α-helices α 1 (residues 53-56), α 2 (residues 119-123), α 3 (residues 124-130).

Additionally, the N-terminal region, consisting of the strands ß 1- ß 7 and the helix α 1, forms a distinct structural motif called the oligonucleotide/oligosaccharide binding-fold (OB fold). The OB fold of EMAP II has a five stranded Greek-key ß-barrel (strands ß 1- ß 3, ß 4, and ß 7) that is capped by the short helix α 1, which is located between the strands ß 3 and ß 4. The C-terminal region contains the strands ß 8- ß 11, the helices α 2- α 3, and several long loops. This C-terminal region contains longer loops compared to the N-terminal region and does not share a homology to any known structure.

EXAMPLES

The present invention will be more specifically explained by the following example. However, it should be understood that the example is intended to illustrate but not in any manner to limit the scope of the present invention.

<Example 1>

Expression and purification of p43, its N-terminal protein and its C-terminal protein

In order to compare the various cytokine activities, anti-tumor activity and anti-angiogenic activity of p43 and EMAP II, the p43, its N-terminal protein and its C-terminal protein were expressed in a recombinant type, and further separated and purified.

A recombinant vector containing the full-length p43 was prepared as follows according to the conventional method(Park et al., *J. Biol. Chem.* 274:16673-16676). From a plasmid pM338 offered by Kiyataka Shiba in the Cancer Institute in Japan, p43 gene was digested with Nde1 and Xho1, and was subjected to PCR using sense and anti-sense primers of SEQ ID NO:3 and SEQ ID NO:4, respectively under conditions of 95℃ for 1 minute, 58℃ for 1 minute and 72℃ for 1 minute. Additionally, the cDNAs encoding N-terminal and C-terminal were separated by subjecting them to PCR using a pair of primers of SEQ ID NO:5 and SEQ ID NO:6, and SEQ ID NO:7 and SEQ ID NO:8, respectively.

The cDNAs obtained were cloned into a plasmid pET28a(Novagen, Madison, USA) with EcoR1/Sal1, Nde1/Xho1 and EcoR1/Sal1, respectively.

Escherichia coli BL21(DE3) was transformed with the recombinant vector containing these genes, and the transformed E. coli BL21(DE3) was cultured in 100 ml of LB(Luria Broth) medium(1g NaCl, 1g Bacto-trypton, and 0.5g yeast extract) to express the genes in a type of a His-tag fusion protein. The cells expressing the recombinant proteins were recovered and suspended in buffer solution(20mM KH_2PO_4, 500mM NaCl, pH 7.8, 2mM 2-mercaptoethanol), and the suspension was sonicated. The sonicated solution was centrifuged at 25,000g, then the supernatant was obtained. The supernatant was subjected to nickel affinity chromatography to purify the proteins

expressed. As a result of SDS-PAGE analysis of the purified proteins, as shown in Fig. 1, it was observed that p43 having the molecular weight of about 43 kDa, N-terminal protein having the molecular weight of about 21 kDa, and the C-terminal protein(EMAP II) having the molecular weight of about 26 kDa were separated purly.

<Example 2>

Induction of cytokines by p43

In order to verify the induction effect of cytokines of p43 separated in Example 1, the following experiment was performed. Human monocytic THP-1 cells were cultured on RPMI 1640 medium(Gibco BRL) containing 10% FBS, and 50 μg/ml of streptomycin/penicillin, and divided into a 24 well plate in the amount of 2×10^6cells/ml. After two hours, the human monocytic THP-1 cell in each well was treated with p43 and EMAP II at a concentration of 0.5, 5, 50 and 500 nM, respectively. After two hours of treatment, the induction and concentration of IL-6, IL-8 and TNF-α in the treated cells were determined by an ELISA kit(PharMingen) using their respective antibodies.

As shown in Fig. 2, the induction levels of these cytokines by the treatment of p43 at 50 nM were similar to those with EMAP II at 500 nM, indicating that p43 is more potent in the induction of these cytokines. Then, the induction of the secretion of MMP-9 with p43 or EMAP II was determined by gelatin zymography(Birkedal-Hansen et al., *Biochem. Biophys. Res. Commun.* 107:1173-1178, 1982). A sample was prepared by adding SDS-PAGE loading buffer solution into the supernatant of the human monocytic THP-1 cell cultured medium, and subjected to 10% Tris-glycin polyacrylamide zymogram gel electrophoresis. The gel was washed with renaturation buffer solution for 30 minutes, and then with developing buffer solution(10 mmol/l Tris-HCl, pH 7.5, 0.15 mol/l NaCl, 10 mmol/l $CaCl_2$ and 0.02% NaN_3) at room temperature for 30 minutes.

Then, the washed zymogram gel was reacted with the developing buffer solution at 37℃ for 24 hours, and then stained with commassie blue R-250 solution. As shown in Fig. 3, the cells treated with p43 or EMAP II secreted MMP-9 while the untreated cells as a control did not secrete. Additionally, the MMP-9 secretion was more strongly induced by the treatment of p43 compared to EMAP II.

<Example 3>

Chemotaxis test of p43

The present inventors performed the in vitro motility assay of p43 and IMAP II for human melanoma cells, and confirmed that p43 was a more effective chemoattractant inducing macrophages.

The chemotactic activity of p43 and EMAP II was determined by the conventional method in a 48 well micro-chemotaxis chamber using a 8 μm polyvinylpyrrolidine-fre polycarbonate filter (Neuroprobe, Cabin John, USA) (Aznavoorian et al., *J. Biol. Chem.* 271:3247-3254, 1996). This determination was performed tripilicate.

As shown in Fig. 4, the treatment of 1-10 ng/ml of p43 induced chemotaxis strongly while the treatment of EMAP II induced chemotaxis slightly. Thus, it was observed that p43 itself has more effective anti-tumor activity than EMAP II.

<Example 4>

Anti-angiogenic effect of p43

(4-1) Cell culture

BAEC(Bovine aorta endothelial cell) used for determination of anti-angiogenic effect of p43 was collected from the bovine aorta and cultured. The cell was cultured in DMEM medium containing 20% fetal bovine serum under conditions of 37 ℃ and 5%

CO_2.

(4-2) Inhibition of endothelial cell growth by p43

The BAEC cultured in Example (4-1) was treated with p43 at 100 nM, and then was observed with a microscope. As shown in Fig. 5a, growth of the cell treated with p43 was inhibited while the growth of the untreated cell as a control was not inhibited. Additionally, the BAEC was treated with each of full-length p43 (F), p 43 C-terminal peptide (C) and p43 N-terminal peptide (N), and apoptosis was measured with a microscope. As a result, it was observed that the induction of apoptosis by the treatment of full-length p43 (F) was higher than that of p43 C-terminal peptide (C).

(4-3) DNA fragmentation assay

In order to confirm apoptosis induction in a cell treated with p43, DNA fragmentation assay was performed according to the conventional method (Ko, Y.G. et al., J. Immunol. 162:7217-7223, 1999). The BAEC was treated with p43 at 100 nM and recovered after 24 hours. The cell precipitate was treated with 500 μl of dissolving buffer(100mM Tris-HCl, pH 8.5, 5mM EDTA, 0.2M NaCl, 0.2% SDS, 0.2mg/ml proteinase K), and reacted at 37℃ for 24 hours to obtain the chromosomal DNA. The chromosomal DNA was treated with 1.5M NaCl and ethanol to precipitate, and treated with 200 μg/ml of RNase to remove RNA. The RNA-removed chromosomal DNA was developed on 1.8% agarose gel to confirm the DNA fragmentation level. As shown in Fig. 5c, the DNA fragmentation was not occurred in the chromosomal DNA isolated from a control cell without treatment of p43 while it was occurred in the chromosomal DNA isolated from p43 treated cell. Thus, it was observed that apoptosis was progressed in the endothelial cell treated with p43.

(4-4) Determination of caspase-3 activity

In order to confirm the apoptosis induction of p43, BAEC was treated with p43 and then the caspase activity was determined.

BAEC cultured in Example (4-1) was distributed into a 6-well plate, and treated with 100nM of p43 after 24 hours. After 24 hours of treatment, the cell was recovered and treated with 500 μl of cool dissolving buffer(20mM HEPES, pH 7.5, 1mM DTT, 0.1mM EDTA, 0.5% NP-40, 0.1mM PMSF). The cell lysate was centrifuged at 4℃ and 15,000g for 5 minutes, and the supernatant was collected to be used for determination of the caspase activity. The assay buffer(20mM HEPES, pH 7.5, 2mM DTT, 10% glycerol, 100 μM caspase substrate(Ac-YVAD-pNA for Casp-1; Ac-DEVD-pNA for Casp-3; Ac-IETD-pNA for Casp-8; Ac-LEHD-pNA for Casp-9), Calbiochem, USA) was added into 40 μg of the supernatant, and reacted at 30℃ for 2 hours. The nitroaniline isolated by the caspase activity was determined at 405nm with UV-VIS spectrophotometer(Pharmacia Biotech, Sweden). As a result, as shown in Fig. 5d, the cell treated with p43 had higher caspase-3 activity than a control cell, indicating p43 induced apoptosis because the apoptosis can be induced by the caspase-3 activity.

[THE EFFECT OF THE INVENTION]

As explained above, it is shown that the full-length p43 effectively induces cytokines to increase immune responses, and effectively inhibit tumor and angiogenesis in the present invention. Therefore, p43 of the present invention can be used in the immune-enhancing agent, anti-tumor agent or anti-angiogenic agent as an active ingredient.

[WHAT IS CLAIMED IS]

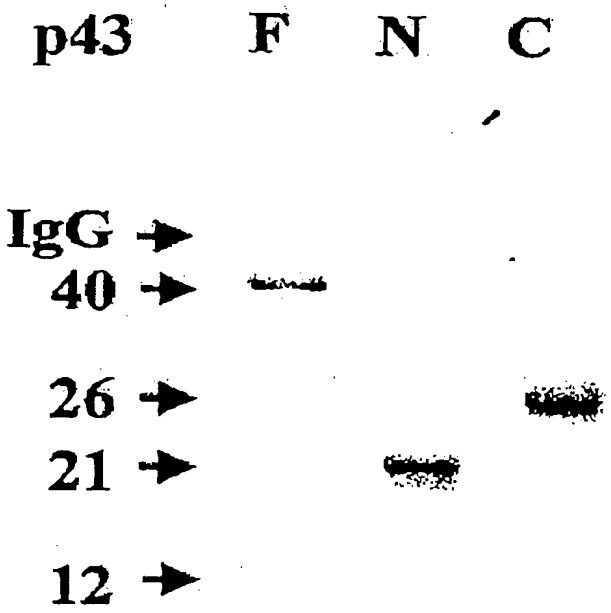
1. An immune-enhancing agent comprising p43 having the amino acid sequence set forth in SEQ ID NO:2 as an active ingredient.

2. An anti-angiogenic agent comprising p43 having the amino acid sequence set forth in SEQ ID NO:2 as an active ingredient.

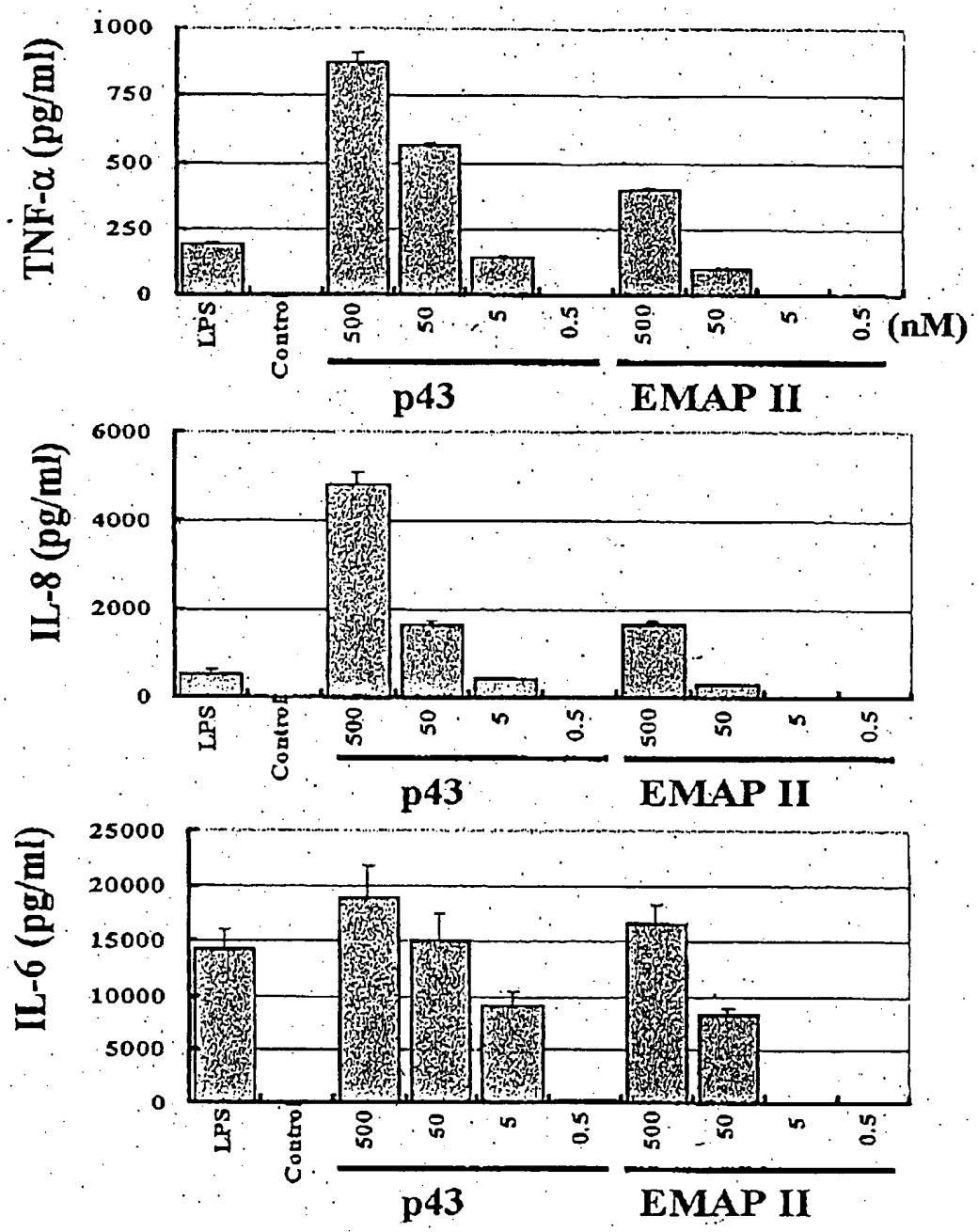
3. An anti-tumor agent comprising p43 having the amino acid sequence set forth in SEQ ID NO:2 as an active ingredient.

[Drawing]

[FIG. 1]



[FIG. 2]


TNF-α (pg/ml)
1000
750
500
250
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Contro
500
50
5
0.5
500
50
5
0.5
(nM)
p43
EMAP II
IL-8 (pg/ml)
6000
4000
2000
0
LPS
Contro
500
50
5
0.5
500
50
5
0.5
p43
EMAP II
IL-6 (pg/ml)
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20000
15000
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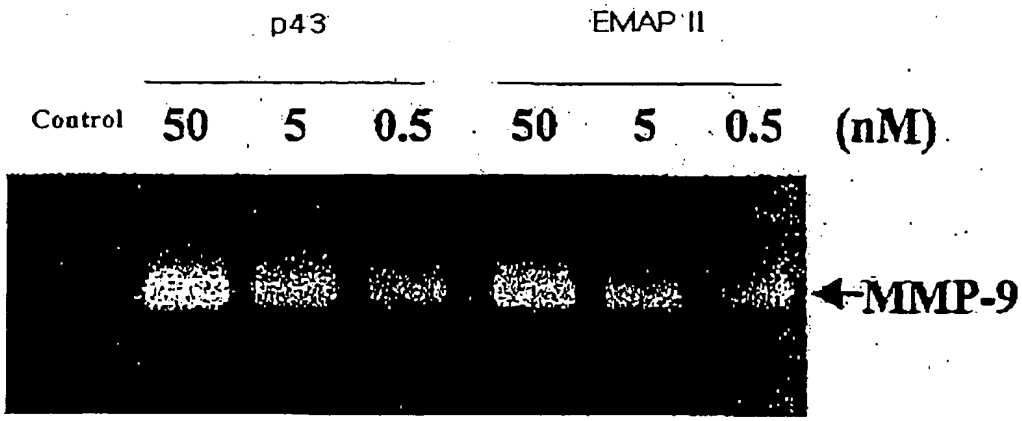
EMAP II

[FIG. 3]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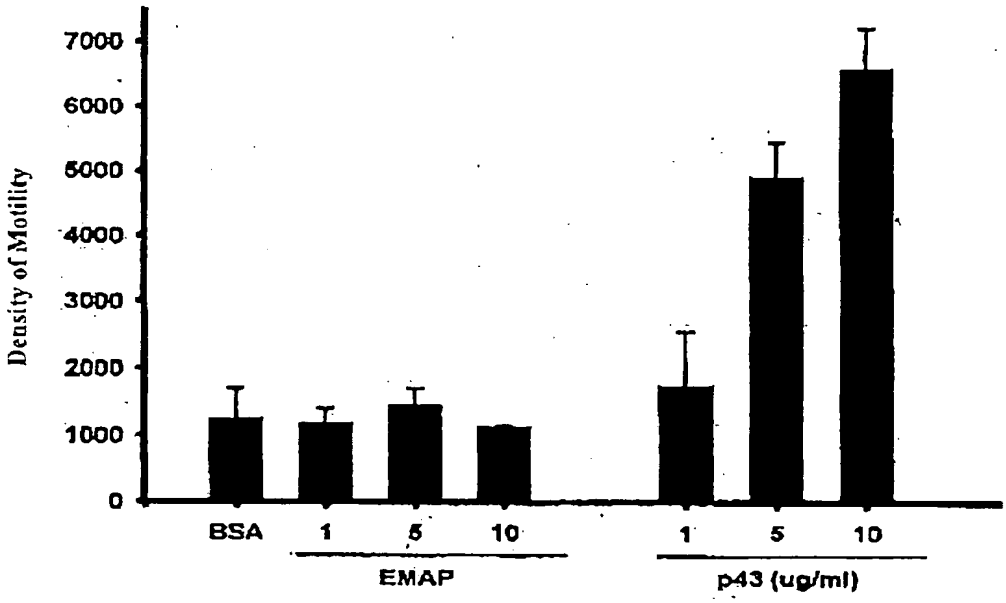
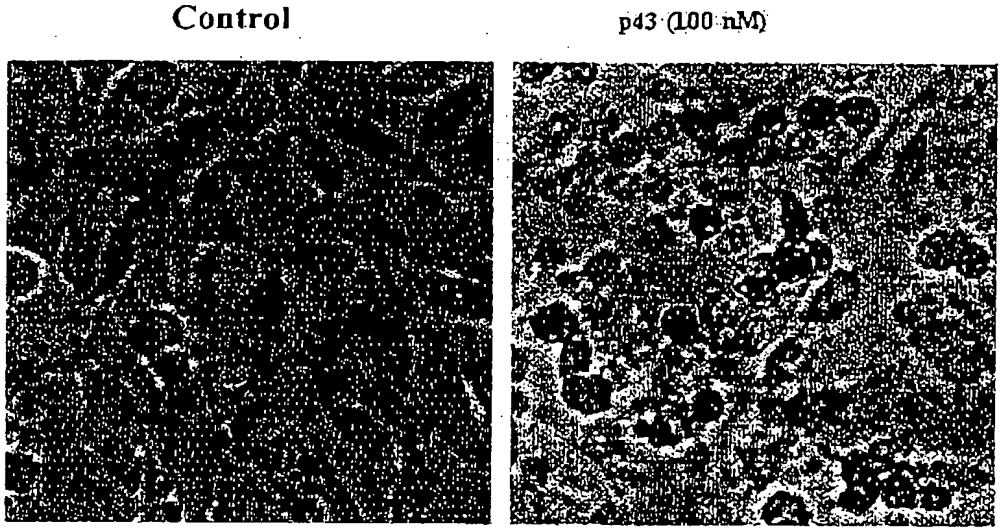
MMP-9

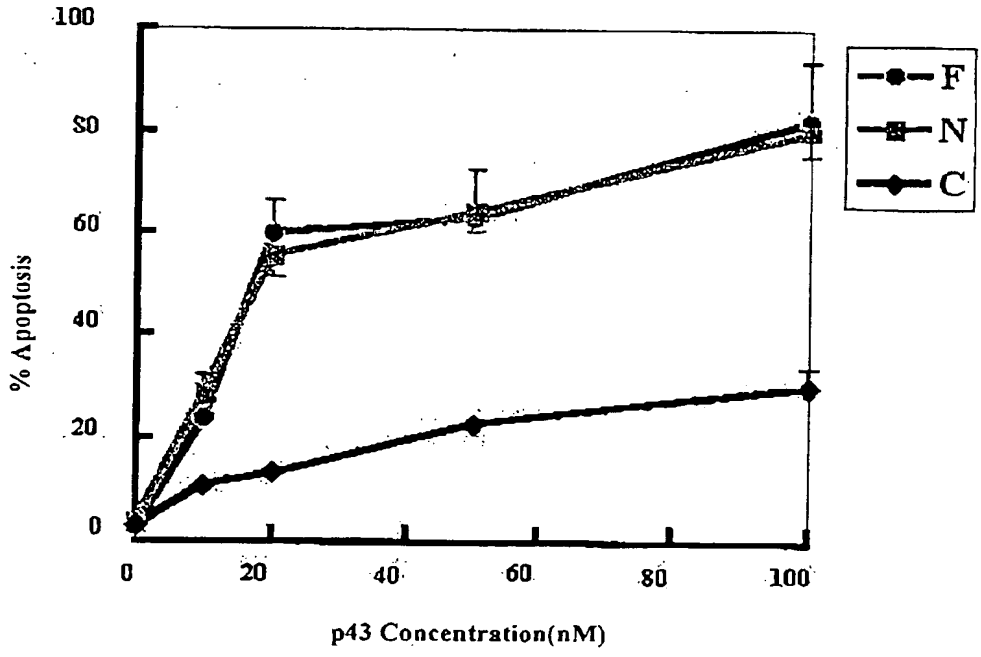
[FIG. 4]


Density of Motility
7000
6000
5000
4000
3000
2000
1000
0
BSA
1
5
10
EMAP
1
5
10
p43 (ug/ml)

[FIG. 5a]


Control
p43 (100 nM)

[FIG. 5b]


100
80
60
40
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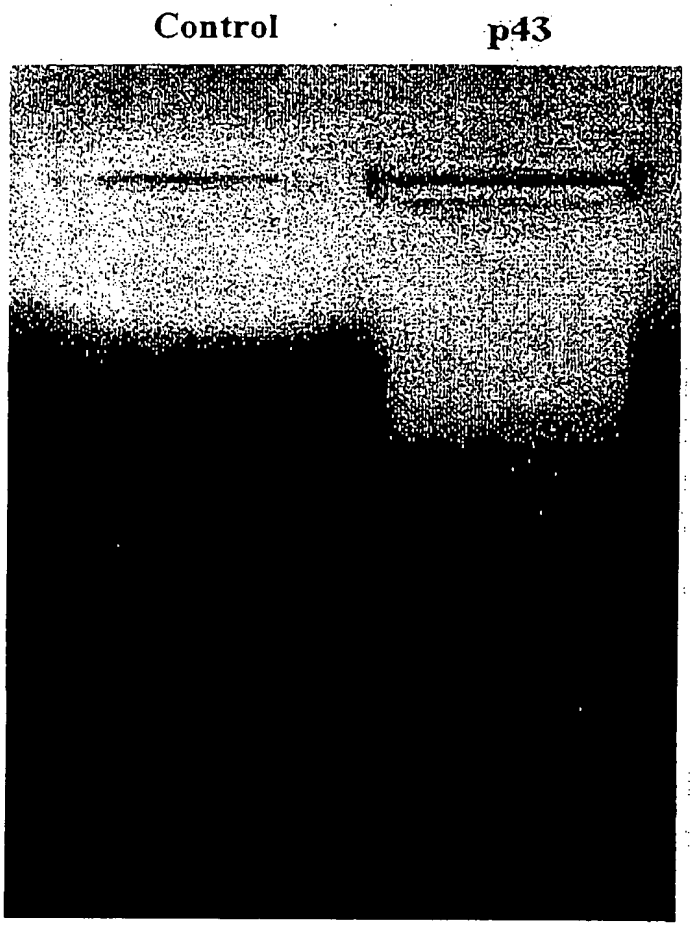
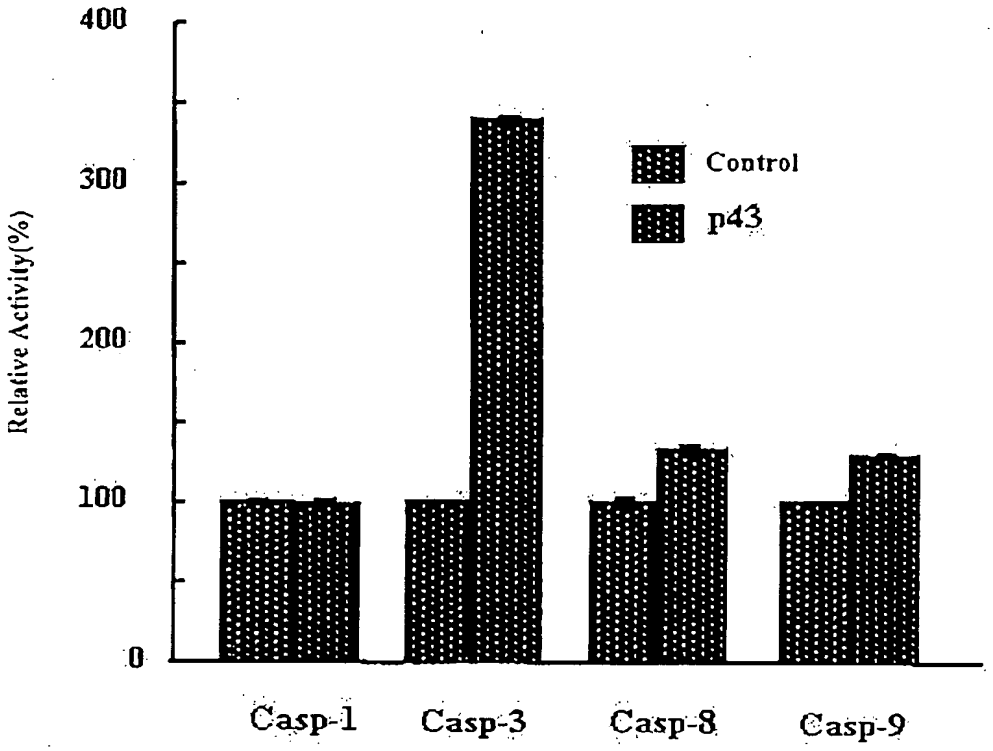
% Apoptosis
0
20
40
60
80
100
p43 Concentration(nM)
F
N
C

[FIG. 5c]



[FIG. 5d]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Control
p43
Casp-1
Casp-3
Casp-8
Casp-9

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Glu Ala Asp Gln Ile Ile Glu Tyr Leu Lys Gln Gln Val Ser Leu Leu
            20   25   30

Lys Glu Lys Ala Ile Leu Gln Ala Thr Leu Arg Glu Glu Lys Lys Leu
35 40 45
Arg Val Glu Asn Ala Lys Leu Lys Lys Glu Ile Glu Glu Leu Lys Gln
50 55 60
Glu Leu Ile Gln Ala Glu Ile Gln Asn Gly Val Lys Gln Ile Ala Phe
65 70 75 80
Pro Ser Gly Thr Pro Leu His Ala Asn Ser Met Val Ser Glu Asn Val
85 90 95
Ile Gln Ser Thr Ala Val Thr Thr Val Ser Ser Gly Thr Lys Glu Gln
100 105 110
Ile Lys Gly Gly Thr Gly Asp Glu Lys Lys Ala Lys Glu Lys Ile Glu
115 120 125
Lys Lys Gly Glu Lys Lys Glu Lys Lys Gln Gln Ser Ile Ala Gly Ser
130 135 140
Ala Asp Ser Lys Pro Ile Asp Val Ser Arg Leu Asp Leu Arg Ile Gly
145 150 155 160
Cys Ile Ile Thr Ala Arg Lys His Pro Asp Ala Asp Ser Leu Tyr Val
165 170 175
Glu Glu Val Asp Val Gly Glu Ile Ala Pro Arg Thr Val Val Ser Gly
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195 200 205
Leu Leu Cys Asn Leu Lys Pro Ala Lys Met Arg Gly Val Leu Ser Gln
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Ala Met Val Met Cys Ala Ser Ser Pro Glu Lys Ile Glu Ile Leu Ala
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Pro Gly Glu Pro Asp Lys Glu Leu Asn Pro Lys Lys Lys Ile Trp Glu
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Gln Ile Gln Pro Asp Leu His Thr Asn Asp Glu Cys Val Ala Thr Tyr
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Thr Met Ser Asn Ser Gly Ile Lys
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